Molecular characterisation of multi-drug resistant Escherichia coli of bovine origin

Anes, João ; Van Nguyen, Scott ; Eshwar, Athmanya K ; McCabe, Evonne ; Macori, Guerrino ; Hurley, Daniel ; Lehner, Angelika ; Fanning, Séamus

Abstract: Antimicrobial resistance reported in bacteria of animal origin is considered a major challenge to veterinary public health. In this study, the genotypic and phenotypic characterisation of twelve Escherichia coli isolates of bovine origin is reported. Twelve bacterial isolates of animal origin were selected from a previous study based on their multidrug resistant (MDR) profile. Efflux pump activity was measured using ethidium bromide (EtBr) and the biofilm forming ability of the individual strains was assessed using a number of phenotypic assays. All isolates were resistant to tetracyclines and a number of isolates expressed resistance to fluoroquinolones which was also confirmed in silico by the presence of these resistance markers. Amino acid substitutions in the quinolone resistance-determining regions were identified in all isolates and the presence of several siderophores were also noted. Whole genomesequence (WGS) data showed different STs that were not associated with epidemic STs or virulent clonal complexes. Seven isolates formed biofilms in minimal media with some isolates showing better adaptation at 25 °C while others at 37 °C. The capacity to efflux EtBr was found to be high in 4 isolates and impaired in 4 others. The pathogenicity of three selected isolates was assessed in zebrafish embryo infection models, revealing isolates CFS0355 and CFS0356 as highly pathogenic. These results highlight the application of NGS technologies combined with phenotypic assays in providing a better understanding of E. coli of bovine origin and their adaptation to this niche environment.

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Molecular characterisation of multi-drug resistant *Escherichia coli* of bovine origin

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Highlights

- 12 bovine isolates out of an Irish collection were sequenced and sequencing data revealed that sequence types (ST) associated with cattle were not associated with epidemic ST or virulent clonal complexes
- Every sequenced isolate showed mutations within *gyrA*, conferring resistance to fluoroquinolones
- These isolates also show hetereogeneity in biofilm formation
- Zebrafish embryo infection studies show that *E. coli* isolates of bovine origin are pathogenic

Abstract-

Antimicrobial resistance reported in bacteria of animal origin is considered a major challenge to veterinary public health. In this study, the genotypic and phenotypic characterisation of twelve *Escherichia coli* isolates of bovine origin is reported.

Twelve bacterial isolates of animal origin were selected from a previous study based on their multidrug resistant (MDR) profile. Efflux pump activity was measured using ethidium bromide (EtBr) and the biofilm forming ability of the individual strains was assessed using a number of phenotypic assays.

All isolates were resistant to tetracyclines and a number of the isolates expressed resistance to fluoroquinolones which was also confirmed *in silico* by the presence of a number of these resistance markers. Amino acid substitutions in the quinolone resistance-determining regions were identified in all isolates and the presence of several siderophores were also noted. WGS data showed different STs that were not associated with epidemic STs or virulent clonal complexes.

Seven isolates formed biofilms in minimal media with some isolates showing better adaptation at 25 °C while others at 37°C. The capacity to efflux EtBr was found to be high in 4 isolates and impaired in 4 others.

The pathogenicity of three selected isolates was assessed in zebrafish embryo infection models, revealing isolates CFS0355 and CFS0356 as highly pathogenic.

These results highlight the application of NGS technologies combined with phenotypic assays in providing a better understanding of *E. coli* of bovine origin and their adaptation to this niche environment.

Keywords: antimicrobial resistance; *Escherichia coli*; biofilm; whole genome analysis

Introduction-

Intensive use of antimicrobial compounds in agricultural and veterinary settings has been documented to contribute to the emergence of resistance phenotypes among bacteria of animal origin (Economou and Gousia, 2015; Wepking et al., 2017). Even in the absence of direct antimicrobial pressure, the spread of resistance determinants continues to occur being mainly associated with horizontal exchange of mobile genetic elements (MGE) among different bacterial species in these niche environments (Sjölund et al., 2008; Poeta et al., 2009; Frye et al., 2011). The emergence of antimicrobial resistance in food-producing animals is of particular concern as these zoonotic bacteria pose a risk to human health *via* the food chain (Hammerum and Heuer, 2009). Among the pathogens that are of importance to human health, *Salmonella* species and *E. coli* are of relevance due to their propensity to contribute to the dissemination of antimicrobial resistance markers (Laufer et al., 2014; Dahms et al., 2015; Robertson et al., 2016).

Bovine animals are a major reservoir for *E. coli*, in particular, the Shiga-toxin producing *E. coli* (STEC) O157:H7 (Ferens and Hovde, 2011; Bono et al., 2012). Understanding how these and other serotypes of *E. coli* adapt to the host environment, bovine and human alike, and what drives the acquisition of antimicrobial resistance is of primary concern. By extending our knowledge of these processes it may be possible to devise strategies to reduce the spread of antimicrobial resistance *via* the food chain. Moreover, the transmission of antimicrobial resistance genes between pathogenic and commensal bacteria poses a threat that can no longer be ignored (Szmolka and Nagy, 2013; Kheiri and Akhtari, 2016; Samei et al., 2016).

The paucity of new antimicrobial compounds being delivered from drug development pipelines is an important public health challenge. Thus, alternative strategies must be considered and developed as a means of controlling the spread of antimicrobial resistance. Some of these

approaches have been applied to reverse the dissemination of these markers. However, the successful adoption of these approaches has had only limited success to date (Lomovskaya and Bostian, 2006).

In this work, a subset of *E. coli* bovine isolates from a collection of Irish *E. coli* of animal origin previously described by Karczmarczyk et al., 2011 were selected for further characterisation. These isolates were assayed for biofilm and pellicle formation, antibiotic resistance, and efflux pump activity in conjunction with whole genome sequencing data.

Materials and Methods-

Bacterial study isolates and reagents

Twelve *E. coli* veterinary clinical isolates of bovine origin obtained in 2007 from the UCD Veterinary Hospital (Karczmarczyk et al., 2011), in Dublin were further investigated (Table S1). *E. coli* ATCC™ 25922 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC™14028 were included as reference strains in all phenotypic assays.

Mueller-Hinton (MH) broth and MH agar, Luria-Bertani (LB) broth, phosphate buffered saline (PBS) and all powder-based antimicrobial compounds, apart from moxifloxacin, were obtained from Sigma-Aldrich (Darmstadt, Germany). Moxifloxacin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All the diffusion disk antibiotics were purchased from Oxoid (Thermo Scientific, Waltham, MA, USA).

Antibiotic susceptibility test (AST)

Susceptibility of each isolate to a panel of antimicrobial compounds was determined by disk diffusion according to the Clinical and Laboratory Standards Institute (CLSI) protocols. Bacterial overnight cultures were diluted in sterile 0.85% NaCl suspension medium (bioMérieux, Marcy-l'Étoile, France) to 0.5 McFarland standard and spread using a cotton swab. Disks containing amikacin (AK – 30 µg), aztreonam (ATM – 30 µg), cefepime (FEP – 30 µg), cefotaxime (CTX – 5 µg), ceftazidime (CAZ – 10 µg), chloramphenicol (C – 30 µg), ciprofloxacin (CIP – 5 µg), doripenem (DOR – 10 µg), doxycycline (DO – 30 µg), ertapenem (ETP – 10 µg), gentamicin (CN – 10 µg), imipenem (IPM – 10 µg), levofloxacin (LEV – 5 µg), meropenem (MEM – 10 µg), minocycline (MH – 30 µg), moxifloxacin (MXF – 5 µg), nalidixic acid (NA – 30 µg), norfloxacin (NOR – 10 µg), tetracycline (TET – 30 µg), ticarcillin (TIC – 75 µg), ticarcillin-clavulanic acid (TIM – 85 µg), tigecycline (TGC – 15 µg) and trimethoprim-

sulfamethoxazole (SXT – 25 μg) were included. Inoculated plates, containing the disks above were incubated at 37°C and zone inhibition measured after 16–18 h. Susceptibility or resistance was determined by CLSI guidelines, M100-S23 (CLSI, 2018). In the case of moxifloxacin and tigecycline the breakpoints used were those from the European Committee on Antimicrobial Susceptibility Testing - EUCAST (2016) (http://www.eucast.org/clinical_breakpoints/). This assay was performed in triplicate for each clinical isolate.

Genomic and plasmid DNA purification

Genomic DNA was purified from overnight cultures using the UltraClean® Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) according to manufacturer's instructions. Large plasmid purification was carried out using S1-nuclease (Promega, Madison, WI) followed by pulsed-field gel electrophoresis (PFGE). *E. coli* 39R 861 and *E. coli* V517 were included as controls. Briefly, the procedure included a lysis step for the bacterial cells, previously embedded in agarose plugs followed by digestion with 8 U S1-nuclease at 37°C for 45 min. Finally, each plasmid sample was then resolved by PFGE in a Chef-Mapper® XA System (Bio-Rad, Hercules, CA) at 14°C, with a switch time setting between 1 and 12 s, at 6 V/cm on a 120° angle in 0.5X TBE buffer for 18 h in a 0.8% [w/v] agarose gel and stained with SYBR green. The approximate molecular mass of plasmids was determined by comparing band molecular sizes with the two control isolates and by using *Salmonella* Braenderup H9812 previously digested with XbaI (Wang et al., 2013).

Bacterial whole genome sequencing

Genomic libraries were prepared using the NEBNext Ultra II (New England Biolabs, Ipswich, MA) for each of the twelve *E. coli* sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). The quality of the reads was assessed using FastQC (version 0.11.5). Error correction was performed using BFC (version r181). A relaxed quality trim was performed using Trimmomatic (version 0.36) before *de novo* assembly using SPAdes (version 3.9.1). The quality of the subsequent assemblies was assessed using Bandage (version 0.8.1) and QUAST (version 5.2). Prokka 1.13.3 was used to annotate the genomes for use in Roary (3.11.2) to determine the pangenome. Roary pangenome results were visualised in UpSetR (https://github.com/hms-dbmi/UpSetR).

Antimicrobial resistance-encoding genes were identified using Resfinder 3.2 (accessed 26-11-2019) (https://cge.cbs.dtu.dk/services/ResFinder/). Plasmid replicon typing was performed using PlasmidFinder 1.3 (https://cge.cbs.dtu.dk//services/PlasmidFinder/). Antibacterial biocide and metal resistance genes were extracted by querying the BacMet 1.1 database (http://bacmet.biomedicine.gu.se) and virulence genes were identified by comparison with the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/). Sequences from these databases were identified within the genomes of all isolates using BLAST+ (version 2.5.0) and Biopython (version 1.68). Multilocus sequence typing (MLST) and serotyping was performed *in silico* using the SRST2 tool. Phylogrouping was performed using ClermonTyper (Beghain et al., 2018).

Raw sequencing data is deposited in the SRA under study accession PRJEB33643 (Supplementary Table 1).

Amino acid substitutions in the quinolone resistance-determining regions (QRDR), global regulatory-encoding genes, RND efflux pump *acrAB-tolC*, outer membrane proteins, biofilm, curli and cellulose genes were identified by comparing the genes against the reference strain *E. coli* K-12 MG1655

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC values for cefotaxime (CTX), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (CN), imipenem (IMP), kanamycin (KM), moxifloxacin (MXF), nalidixic acid (NAL), and tetracycline (TET) were measured by two-fold broth microdilution in a 96-well microtiter plates as described previously (Anes et al., 2017).

Biofilm formation assay, Congo red, calcofluor staining and pellicle formation

Biofilm formation was examined under defined growth parameters. M9 minimal media (containing NH_4Cl [1.9 mM], Na_2HPO_4 [42.3 mM], KH_2PO_4 [22 mM], NaCl [8.56 mM], $MgSO_4$ [2 mM], $CaCl_2$ [0.1 mM], and glucose 0.1% [w/v] was used to enable an assessment of biofilm formation and cultures were incubated at 25 and 37 °C respectively at 24 and 48 hours.

Initially, overnight cultures were adjusted to $OD_{600\,nm}$ 0.3 with fresh media, and 200 µL of this bacterial cell suspension was dispensed across a 96-well microtiter plate. The plates were

incubated statically for 24 and 48 hours at temperatures of 25 and 37°C respectively. *Salmonella* Typhimurium ATCC™14028 was used as a positive control for biofilm formation. A crystal violet assay was carried out to quantify the mass of biofilm formed as before (Stepanović et al., 2007).

The expression of curli fimbriae was assessed by examining the colony morphology of the *E. coli* isolates on Congo red agar. Calcofluor agar plates were used to assess the ability of each isolate to produce cellulose (Anes et al., 2017). The Congo red agar and the calcofluor plates were spotted with 3 µL aliquots of overnight bacterial culture grown in MH broth and incubated for 72 h at 25 and 37°C after which colony morphology was inspected. The calcofluor plates were visualised under a 366-nm UV light source in order to detect the binding of the fluorescent dye to any cellulose produced. *Salmonella* Typhimurium ATCC™14028 was included as a control and both assays were carried out in triplicate for each isolate.

For pellicle formation, 50 µL from an overnight culture in MH broth was used to inoculate 5 mL MH broth, which was then incubated statically for 96 h at 25 and 37°C after which it was then inspected for the formation of a pellicle at the air-broth interface (Zogaj et al., 2001). This assay was repeated on two independent occasions and results were registered as present or absent.

Assessment of efflux pump activity by fluorimetry using ethidium bromide (EtBr)

Ethidium bromide (EtBr) was used as a marker to assess the efflux capacity of each clinical isolates as previously described (Anes et al., 2017). In brief, bacterial isolates were grown to mid-$\log_{10}$ phase and washed twice with PBS then the $OD_{600\ nm}$ was adjusted to 0.6. Cells were then subsequently incubated in PBS in the presence of EtBr (50 µM) and 100 µM of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) at 25 °C for 1 h. Afterwards, the cells were centrifuged for 16,000 x g for 3 min. and the cells resuspended in **i)** PBS; **ii)** PBS with 50 mM glucose and **iii)** PBS with 100 µM CCCP. Aliquots of 0.1 ml were transferred into a 96-well microtitre plate and EtBr fluorescence was measured in a Fluoroskan Ascent FL (Thermo Scientific, Waltham, MA) with excitation and emission wavelengths of 518- and 606-nm, respectively. Fluorescence emissions were acquired in cycles of 60 seconds, for a duration of 50 min. at 37 °C. Each assay was performed in triplicate. The efflux of Etbr was presented as relative fluorescence which was obtained by comparing the fluorescence observed for the bacterial cells in the presence or absence of glucose and the control in which the cells are

exposed to conditions of minimum efflux (in the absence of glucose and in the presence of CCCP).

Zebrafish ethics statement

Experiments were carried out until 72 hours post infection (hpi) and at the end of the experiments, embryos that were alive were euthanized with an overdose of 4 g/L buffered tricaine. Since pain sensitivity has not developed at these earlier stages (5 days post fertilization, dpf), this is not regarded as a painful technique. The maximum age reached by the embryos during experimentation was 120 hpf (72 hpi) for which no license is required from the Swiss cantonal veterinary office. This research was conducted with approval (NO 216/2012) from the Veterinary Office, Public Health Department, Canton of Zurich (Switzerland) allowing experiments with zebrafish embryos and larvae. The methods applied were carried out following approved guidelines.

Zebrafish lines and husbandry

Zebrafish (*Danio rerio*) strains used in this study were *wik* lines. Adult fish were kept at a 14/10 hours light/dark cycle at a pH of 7.5 and 27°C. Eggs were obtained from natural spawning between adult fish which were set up pairwise in individual breeding tanks. Embryos were raised in petri dishes containing E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) supplemented with 0.3 µg/mLmethylene blue at 28°C. From 24 hpf, 0.003% 1-phenyl-2-thiourea (PTU) was added to prevent melanin synthesis. Staging of embryos was performed as previously described (Kimmel et al., 1995).

Microinjection experiments

Injections were performed using borosilicate glass microcapillary injection needles (Science Products, 1210332, 1 mm O.D. x 0.78 mm I.D.) and a PV830 Pneumatic PicoPump (World Precision Instruments). Thirty-six-hour-post-fertilization (hpf) embryos were manually dechorionated and anesthetized with 200 mg/L buffered tricaine (Sigma, MS-222) prior to injections. Subsequently embryos were aligned on an agar plate and injected with 100 CFU (range 86 - 128 CFU) in 1-2 nL volume of a bacterial suspension in DPBS directly into the blood circulation (caudal vein). Prior to injection the volume of the suspension was adjusted

by injecting a droplet into mineral oil and measuring its approx. diameter over a micrometer scale bar. The number of CFUs injected was determined by injection of bacterial suspension into a DPBS droplet on an agar plate. Following injections infected embryos were allowed to recover in a petri dish with fresh E3 medium for 15 min. To follow infection kinetics and for survival assays, embryos were transferred into 24-well plates (one embryo per well) in 1 mL E3 medium per well, incubated at 28°C and observed for signs of disease and survival under a stereomicroscope twice a day. For survival assays after infection, the number of dead larvae was determined visually based on the absence of a heartbeat. Kaplan Meier survival analysis was done with GraphPad Prism 7 (GraphPad Software, United States). Experiments were performed at least three times, unless stated otherwise.

Results

Phenotypic characterisation of the bacterial isolates

A subset of twelve *E. coli* isolates of bovine origin, from a clinical collection of twenty animal isolates obtained from the UCD Veterinary Hospital in Dublin in 2007 (Karczmarczyk et al., 2011) were further characterised. Their MDR profile was previously determined for some of the antimicrobial compounds selected (Karczmarczyk et al., 2011). In this present study, the antimicrobial drug panel was extended to 21 compounds with representatives of each antimicrobial class. The results presented in **Figure 1** shows the resistance profiles of these twelve *E. coli*. The isolates clustered based on their resistance profile and all were resistant to quinolones, fluoroquinolones, and tetracyclines. Based on the antimicrobial resistance profile and previously published data *E. coli* CFS0344, CF0345, CFS0347 to CFS051, CFS0354-CFS0356, CFS0359 and CFS0360 were selected for further genotypic and phenotypic studies.

The antimicrobial resistance profile of the selected isolates was further confirmed by broth microdilution with several antimicrobial agents representative of different classes. Minimum inhibitory concentration (MIC) values were determined (**Table 1**) and the results showed that for the majority of the isolates, their resistance profile matched the results obtained by disk diffusion. In the case of *E. coli* CF0344, CFS0345 and CFS0350 an imipenem resistance phenotype was noted, for the first two of these and an intermediate phenotype for the remaining isolate, a pattern that was not captured by disk diffusion assay. All isolates determined to be resistant to more than 3 classes of compounds were defined as being multi-drug resistant

(MDR). Minimum bactericidal concentration (MBC) values (**Table S2**) were similar to those obtained for MIC measurements.

Determination of the efflux pump activity by fluorimetry for *E. coli* cultured from bovine animals

The ability of these bacterial isolates to efflux chemical compounds from the cell was assessed by fluorimetry. In order to determine the functional efflux activity of the bacterial isolates, cells were pre-loaded with ethidium bromide and fluorescence measured over a period of 50 minutes. EtBr, when bound to cellular components, fluoresces with more intensity and thus cells with active efflux pumps will appear less fluorescent due to dye extrusion. To explore another feature of the efflux pump activity, previously loaded bacterial cells were incubated in the presence of glucose and/or the proton motive force (PMF) uncoupler CCCP. **Figure 2** shows the differences observed between the isolates studied.

In the presence of PBS alone all isolates exhibited approximately the same efflux capacity. The presence of CCCP prevented efflux of EtBr, leading to minor decreases in the intensity of the fluorescence detected with similar effects for all isolates. When glucose was added to these cells (50 mM final concentration), all were able to extrude EtBr at an increased rate. In the presence of glucose, *E. coli* CFS0354, CFS0355, CFS0356 and CFS0359 had reduced efflux activity compared to the other isolates.

Biofilm formation and associated morphotypes

The potential to form biofilms, confers on bacteria an extra layer of protection against the external environment, including effects of antimicrobial agents and other environmental stresses. All study isolates were tested for their ability to form biofilms under stressful conditions at 25 °C (commonly associated with environmental temperatures) and at 37 °C (associated with human/animal body temperature) using the crystal violet method. The results of these assays are presented in **Figure 3**.

Results indicated that half of the isolates (*E. coli* CFS0345, CFS0351, CFS0354, CFS0359 and CFS360) were unable to form robust biofilms under the *in vitro* conditions tested. *E. coli* CFS0347, CFS0348 and CFS0349 produced stronger biofilms at 25 °C. In contrast, *E. coli* CFS0350, CFS355 and CFS356 produced more robust biofilms when grown at 37 °C.

The isolates were also assessed for their ability to form a pellicle at the air-liquid interface (supplementary **Table S3** and **Table S4**). Only *E. coli* CFS0349 was capable of forming a pellicle at 25 °C. At 37 °C five *E. coli* isolates (including CFS0350, CFS0351, CFS0354, CFS0355 and CFS0356) could form air-liquid interface pellicles.

The expression of curli fimbriae and the production of cellulose, involved in the formation of biofilms was measured using agar plates incubated at 25- and 37-°C and containing Congo red and calcofluor dyes that stain these corresponding features. The results are shown in supplementary **Table S3** and **Table S4** and the associated morphotypes described in each case by reference to *Salmonella enterica* serovar Typhimurium ATCC™14028, an isolate previously reported to produce a RDAR morphotype.

At 25 °C only 3 *E. coli* (CFS0347, CFS0348 and CFS0349) expressed the RDAR morphotype. Two of the collection isolates *E. coli* CFS0355 and CFS0356 elaborated a smooth and white (SAW) morphotype which is characterised by the absence of curli fimbriae and lacking cellulose (Zogaj et al., 2001). The remaining isolates produced an intermediate morphotype that in this case, is defined as red and smooth (RAS). Two of the study isolates, *E. coli* CFS0347 and CFS0348 were determined to produce cellulose. The remaining isolates were considered negative for the expression of this phenotype. The results recorded at 37 °C did not show any typical Congo red morphotypes that would suggest the expression of curli fimbriae. The majority of the isolates produced a SAW morphotype. The expression of cellulose was lacking in all of the isolates when grown at 37 °C.

Characterisation of the genomes of the selected *E. coli* isolates

Genomic DNA was purified and sequenced using the Illumina Miseq platform. An overview of the assembly metrics is presented in supplementary **Table S5** and a visualisation of the pangenome in supplementary **Figure S1**.

Multi-locus sequence types (MLST) were inferred from the genomic sequences of 7 housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA, recA*) according to the published MLST scheme (Wirth et al., 2006). Sequence type results and genomic serotyping are shown in **Table 2**. The majority of the isolates belong to the sequence type ST10 (n=4). *E. coli* CFS0355 and CFS0356 belonged to ST540, whereas the *E. coli* isolates CFS0359 and CFS360 were found to be ST744 types. Two other *E. coli* CFS0347 and CFS0349 were identified as ST90 and ST88 respectively. *E. coli* isolate CFS0348 was identified as ST6901 and CFS0350 is ST23.

According to the ClermonTyper scheme, the majority of the isolates were phylogroup A (**Table 2**). CFS0347, CFS0349, and CFS0350 were phylogroup C while CFS0348 is the lone phylogroup D isolate (**Table 2**).

Genomic-based serotyping revealed that serotype O89:H9 was common among these isolates of types, ST10 and ST744. Serotype O9:H30 was associated with the isolates of the sequence type, ST540. The remaining isolates presented diverse serotypes (see **Table 2**).

Acquired antimicrobial resistant genotypes were extracted from these genome sequences and organised in classes has shown in **Table 2**. More detailed information describing the corresponding antimicrobial resistance-encoding genes are included in supplementary **Table S6.**

Results show that all bacterial isolates were positive for genes that conferred resistance to β -lactam antibiotics. The *bla*$_{\text{TEM}}$ gene was identified in 11 of the 12 isolates. Only one, *E. coli* CFS0347, contained a different β -lactam resistance gene, *bla*$_{\text{CMY}}$. Genes conferring resistance to different members of the aminoglycoside class were found in all isolates and were reflected by their antibiotic resistance phenotypes obtained by AST (**Table S6** and **Figure 1**).

Resistance to tetracycline observed among these isolates was further supported by the presence of the genes *tet*(34), *tet*(A), *tet*(B) and *tet*(C) identified among all isolates. Chloramphenicol resistance was also confirmed by the presence of phenicol resistance-encoding genes in 10 of the 12 isolates. Where isolates were previously determined to be susceptible to this drug, as in the case of *E. coli* CFS0355 and CFS0356, no resistance markers were detected. No acquired resistance gene(s) were identified for fluoroquinolones and carbapenems. The presence of quaternary ammonium resistance-encoding gene *qacEΔ1* was confirmed in 10 of the 12 *E. coli* isolates with the exception of the isolates *E. coli* CFS0351 and CFS0354.

Isolates containing multiple plasmids, as determined by S1-nuclease PFGE profiling included *E. coli* CFS0355 and CFS0356 both belonging to the ST540 complex. The plasmid incompatibility types identified in these isolates included IncF, IncH and IncX types. Similarly, *E. coli* CFS0359 also was positive for three Inc types, denoted as IncN, IncQ and IncR (**Table 2**).

Virulence factor-encoding genes were extracted using the VFDB database as a reference. Data included in **Table 2** shows the presence of the siderophores enterobactin and aerobactin in all the isolates. Four *E. coli*, denoted as CFS0349, CFS0355, CFS0356, CFS0359 and CFS0360 all contained the salmochelin operon (*iroBCDEN*). *E. coli* CFS0349 and CFS0350 also

contained the yersiniabactin operon. Interestingly, *E. coli* CFS0348 also contained the hemin utilization operon (*chu* genes).

The type I fimbriae operon (*fim*) was detected in 10 out of 12 isolates, with the *E. coli* isolates CFS0355 and CFS0356 indicating the presence of only *fimH*. P fimbriae-encoding genes (*pap*) were only identified in *E. coli* CFS00349, CFS0350 and CFS0351. Other fimbriae types such as *fae, f17d* and *afa* were also identified in the genome sequences of several isolates (**Table S6**).

Other relevant virulence genes such as the *tsh* genes, encoding for the temperature-sensitive hemagglutinin, were identified in *E. coli* CFS0355 and CFS0356. The toxin gene *astA* encoding the enteroaggregative heat-stable toxin 1 (EAST1) was also found in 58% of the isolates (n=7). Type II general secretory protein pathway genes (*gsp*) and type III secretion system effectors were identified in the genomes of *E. coli* CFS0347, CFS0348, CFS0349 and CFS0350.

No major differences in the resistance genotypes to heavy metals was noted among the isolate collection. Interestingly, the copper homeostasis and silver resistance island (CHASRI) that confers resistance to copper under aerobic and anaerobic growth conditions, was identified in *E. coli* CFS0355 and CFS0356.

Amino acid substitutions in the GyrA subunit of DNA gyrase were identified in all *E. coli* study isolates. These were detected within the quinolone-resistance determining region (QRDR) at residue positions 83 and 87. These latter substitutions were previously reported as contributing to the emergence of resistance to quinolones and fluoroquinolones (Fu et al., 2013). *E. coli* CFS0348 also presented an additional substitution at position 678, and the role (if any) of this change is unknown. A single amino acid substitution was identified in GyrB in four isolates (*E. coli* CFS0348, CFS0349, CFS0359 and CFS0360) in each case occurred at different positions. The amino acid sequences of ParC and ParE also showed several substitutions. A complete summary of all amino acid substitutions identified can be seen in **Table S7.**

As a diverse resistance phenotype was identified in the study isolates, it is reasonable to question the contribution of efflux transporter systems. Genomic data was assessed for the presence of mutations/amino acid substitutions in a selected number of these specific genes. Amino acid (AA) substitutions were identified in different positions for AcrB and TolC in *E. coli* CFS0348 and CFS0349. Similarly, AA substitutions in the global regulators MarR and MarB were also noted in *E. coli* CFS0347, CFS0348, CFS0349, CFS0350, CFS0355 and CFS0356. Only one AA substitution was recorded in the Rob-encoding regulator at position

192 for *E. coli* CFS0350. Two AA substitutions were recorded in *E. coli* CFS0348 in the regulator SoxR. Several other amino acid substitutions were identified in the membrane porins, OmpC and OmpF in all the isolates.

In addition, several AA modifications were also identified in the cellulose-encoding operon *bcsABCZ* among the *E. coli* isolates CFS0347, CFS0348, CFS0349 and CFS0350 where all of the corresponding genes possessed modifications. The amino acid sequence of BcsZ was found to be truncated in those isolates belonging to the sequence types, ST10 and ST744. Curli fimbriae-encoding proteins CsgA and CsgB were also found to contain amino acid substitutions that in this case were associated with only one *E. coli* isolate, CFS0348. Other genes within this operon, including *csgC* and *csgD* contained AA substitutions in *E. coli* CFS0347, CFS0348, CFS0349, CFS0350, CFS0355 and CFS0356 (**Table S7**).

Pathogenicity of *E. coli* isolates in a zebrafish model

Three of the isolates (*E. coli* CFS0348, CFS0355 and CFS0356) were selected to test their ability to infect zebrafish based on their genotypes and phenotypes. Survival curves were generated for zebrafish embryos (n = 30 per *E. coli* isolate) infected (100 CFU per embryo) with *E. coli* and monitored over 3 days. Zebrafish embryos rapidly succumbed to the infection with *E. coli* ST540 CFS0355 and CFS0356 strains, while those infected with *E. coli* CFS0348 died later. Survival curves and trends were significantly different (log_{10}-rank test $p < 0.0001$) in **Figure 4.**

Discussion-

The rapid rise in reported antimicrobial resistance is forcing a paradigm shift in how these drugs are applied in various settings. As resistance spreads at a pace that is coupled with a lack of any development of new antimicrobial agents, the need to find workable solutions to this public health challenge has now become urgent. In this paper, a collection of *E. coli* of bovine origin obtained from the UCD Veterinary Hospital were further studied. All were re-tested for their susceptibility to a panel of compounds and subjected to a number of more detailed phenotypic and genotypic investigations. Based on the MLST data obtained, none of the isolates were associated with epidemic or virulent clonal complexes. Extended-spectrum AmpC β-lactamases have been linked to ST23 (Crémet et al., 2010) which highlights how widespread antibiotic resistance is in diverse *E. coli* of animal origin. Genomic-based

serotyping revealed that serotype O89:H9 was common among these isolates of types, ST10 and ST744. Serotype O9:H30 was associated with the isolates of the sequence type, ST540. Nonetheless a diverse phenotypic picture was observed within the study collection.

All of the selected isolates were found to be resistant to fluoroquinolone and tetracycline compounds. When extracted from the whole genome sequence reads, acquired genotypes identified, for the most part, showed that the majority of these isolates possessed the corresponding resistance genes, originally inferred from the phenotypes determined by AST. Resistance to imipenem was not associated with any particular acquired resistance gene. While members of *Enterobacteriaceae* are intrinsically resistant to macrolides, the presence of the *mph(B)* gene in *E. coli* CFS0355 and CFS0356 is notable as previous studies suggest *E. coli* may serve as reservoirs for macrolide resistance genes (Nguyen et al., 2009). The presence of CHASRI in CFS0355 and CFS0356 may be due to the use of copper as an animal feed supplement and could be responsible for the selective acquisition of the heavy metal resistance genes identified here (Staehlin et al., 2016).

Efflux pump activity is an important intrinsic characteristic of bacteria, which aids bacterial adaptation in an environmental niche and this feature is known to contribute to antimicrobial resistance in some cases (Nikaido and Takatsuka, 2009). In Gram-negative bacteria the best-known efflux system is represented by members of the resistance nodulation-cell division (RND) family. This efflux family, and in particular AcrAB-TolC, has been associated with the extrusion of acriflavine, ethidium bromide, fluoroquinolones, tetracyclines, tigecyclines, and many other compounds from the bacterial cytoplasm (Anes et al., 2015). AcrAB-TolC was also shown to play a role in virulence in several pathogenic bacteria (Blair and Piddock, 2009; Pérez et al., 2012; Tsai et al., 2015). *E. coli* CFS0345 CFS0348, CFS0350 and CFS0351 exhibited an active efflux mechanism when compared with the other isolates. This activity could be attributed to the amino acid substitutions identified at different positions in AcrB and TolC sequences in *E. coli* CFS0348 and CFS0349 or AA substitutions in the global regulators including MarR and MarB in *E. coli* CFS0348, CFS0349 and CFS0350.

Among the virulence genes identified, iron scavenging proteins (siderophores) are of importance. Apart from their obvious biological function wherein they participate in scavenging iron, a co-factor essential for several cellular processes, these proteins are considered to be important to facilitate pathogen survival in the host (Ratledge and Dover, 2000; Snyder et al., 2004; Hagan et al., 2010). Salmochelin (present in *E. coli* CFS0349, CFS0355, CFS0356, CFS0359, and CFS0360) and yersiniabactin (CFS0349 and CFS0350) are

known to be associated with invasive *E. coli* serotypes (Henderson et al., 2009). Interestingly, yersiniabactin has been identified in other ST88 previously (Huja et al., 2015). The presence of these siderophore genes highlight the potential pathogenicity of these *E. coli* from bovine origins. The temperature-sensitive hemagglutinin (*tsh*) was identified in *E. coli* CFS350, CFS0355, and CFS0356 and has previously been reported in bovine *E. coli* (Kassé et al., 2016). ST540 CFS0355 and CFS0356 showed higher mortality when compared to ST6901 CFS0348, however all strains were pathogenic in an *in vivo* zebrafish embryo infection model. The presence of the salmochelin siderophores and *tsh* in CFS0355 and CFS0356 may contribute to increased virulence in zebrafish embryos and ST540 *E. coli have* been previously isolated from urinary tract infections in Denmark (Hertz et al., 2016).

Common incompatibility types identified from the genome sequences included IncF and Col types, both of which are frequently detected among Gram-negative bacteria (Carattoli, 2009). IncHI2 plasmids have been implicated for the spread of heavy metal resistance islands in *E. coli* associated with food producing animals (Fang et al., 2016) and the IncHI2 plasmid replicons were detected in *E. coli* CFS0355 and CFS0356.

Five of the *E. coli* (isolates CFS0345, CFS0351, CFS0354, CFS0359 and CFS360) were unable to form biofilms under the laboratory-induced *in vitro* conditions used. Of those isolates capable of forming biofilms (n=7), three appeared to form robust biofilms when cultured at 37 °C, whereas the remaining four isolates formed better biofilms at the environment temperature (25 °C). The RDAR morphotype (indicating the expression of fimbriae and cellulose production) was observed only at 25 °C in 3 *E. coli* (CFS0347, CFS0348 and CFS0349). Several modifications identified within related genes were detected and these included the cellulose biosynthetic-encoding operon *bcsABCZ,* in particular, in *E. coli* CFS0347, CFS0348, CFS0349 and CFS0350. *E. coli* CFS0348 contained multiple substitutions within the corresponding genes encoding curli proteins CsgA and CsgB. Of the remaining proteins of the curli operon, amino acid substitutions in CsgC and CsgD were present in CFS0347, CFS0348, and CFS0350.

Conclusions-

The collection of isolates chosen in this study were from bovine origins. All of the isolates expressed an MDR phenotype and notably, every isolate had mutations within *gyrA* resulting in FQ resistance. The MDR phenotype of these isolates originated from acquired resistance

mechanisms including *bla*$_{TEM}$, *bla*$_{CMY}$, and aminoglycoside genes together with accumulation of point mutations in the QRDR. In addition to genetic changes, *E. coli* CFS0345, CFS0348, CFS0350, and CFS0351 showed increased efflux pump activities resulting in diverse MDR phenotypes. Interestingly no acquired resistance mechanisms against FQ nor carbapenems were detected. A number of different plasmid replicon types were identified, including IncFIB, Col and IncHI2. These plasmids are important vectors for the of horizontal transfer of antimicrobial resistance, heavy metal resistance, and virulence genes such as siderophores.

WGS of twelve of the isolates revealed diverse STs that were not associated with epidemic STs or virulent clonal complexes. Several serotypes were identified in the sequenced *E. coli* of bovine origin including O8:H9, O8:H19, O9:H12, O9:H30, O11:H15, and O89:H9. In addition to ST diversity, these isolates expressed phenotypic heterogeneity in biofilm formation. The differences in biofilm formation at host or environmental temperatures would suggest potential niche adaptation for some of the isolates. Zebrafish infection studies with 3 of the isolates revealed that *E. coli* of bovine origin are pathogenic. The virulence of *E. coli* CFS0355 and CFS0356 (most virulent isolates tested) may be linked with the presence of several encoding siderophores.

These results show that overall, the genomic characterisation reflects the phenomic data. In some cases, however accurate AMR prediction from genomic information can be challenging. These results underly the importance of using NGS technologies in combination with traditional phenotypic methods to corroborate genomic data and to assay for genotypes and phenotypes of bovine *E. coli* suggestive of its potential for niche adaptation.

Conflict of Interest

The authors have nothing to declare.

Acknowledgments

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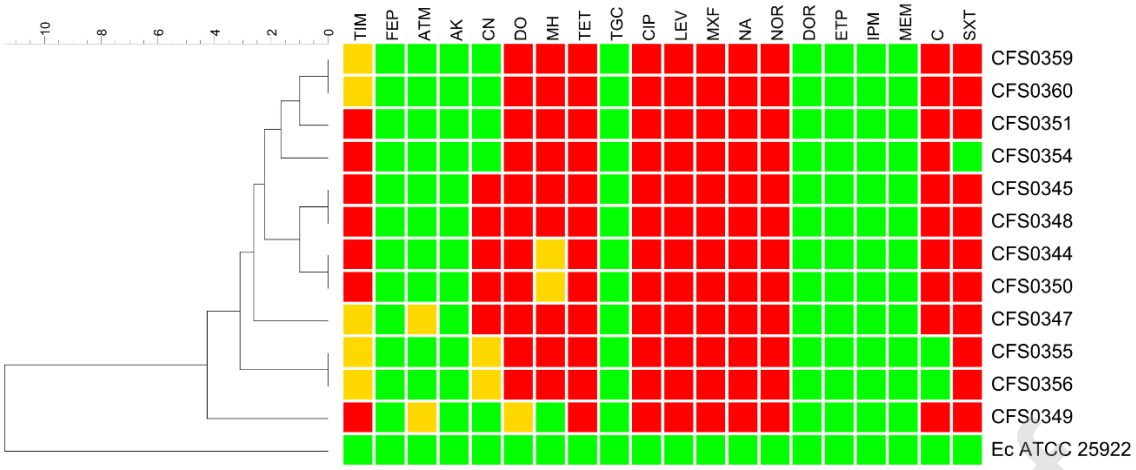

Figure 1. Dendrogram constructed using BioNumerics™ software (ver 7.5, Applied Maths, Sint-Martens-Latem, Belgium), showing the antibiotic resistance profiles detected following disk diffusion assays. The dendrogram shows the clustering based on these resistance profiles. Red coloured cells indicate – resistant phenotypes; yellow – intermediate resistance and green – susceptible phenotypes. Breakpoints were interpreted according to CLSI guidelines 2018 with the following exception: Tigecycline, Moxifloxacin - the breakpoints used in these cases were those published in the EUCAST guidelines 2016. The following abbreviations apply to the listing of compounds within individual drug classes: **TIM**-Ticarcillin-clavulanic acid; **FEP**-Cefepime; **ATM**-Aztreonam; **AK**-Amikacin; **CN**-Gentamicin; **DO**-Doxycycline; **MH**-Minocycline; **TE**-Tetracycline; **TGC**-Tigecycline; **CIP**-Ciprofloxacin; **LEV**-Levofloxacin; **MXF**-Moxifloxacin; **NA**-Nalidixic Acid; **NOR**-Norfloxacin; **DOR**-Doripenem; **ETP**-Ertapenem; **IPM**-Imipenem; **MEM**-Meropenem; **C**-Chloramphenicol and **SXT**-Trimethoprim-Sulfametoxazole.

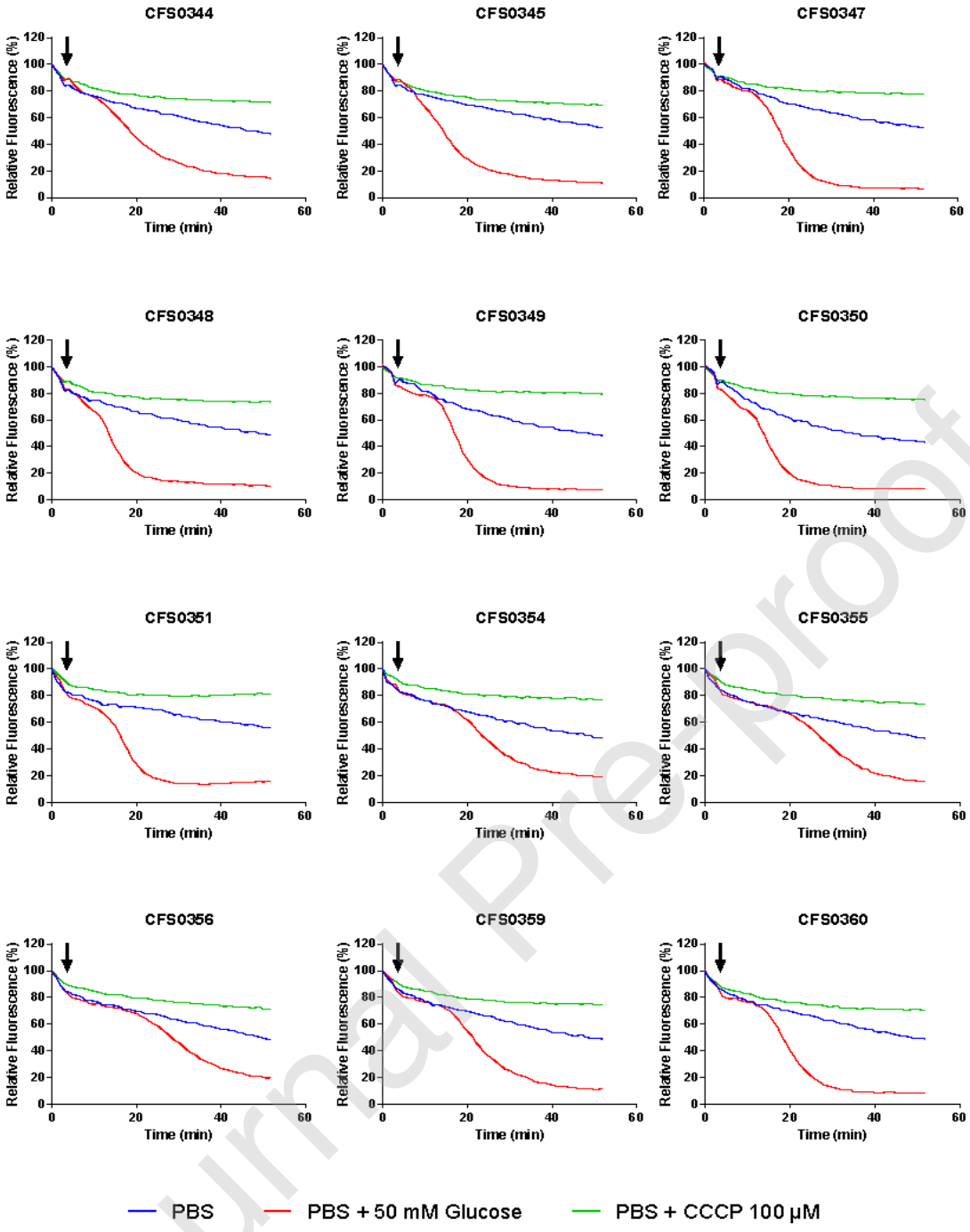

Figure 2. Determination of efflux activity for all 12 *E. coli* by a semi-automated fluorometric method. Isolates were saturated with 50 µM ethidium bromide and efflux measured by fluorimetry at 37 °C for 50 minutes. Arrowheads indicate the time points at which either glucose and/or CCCP were added.

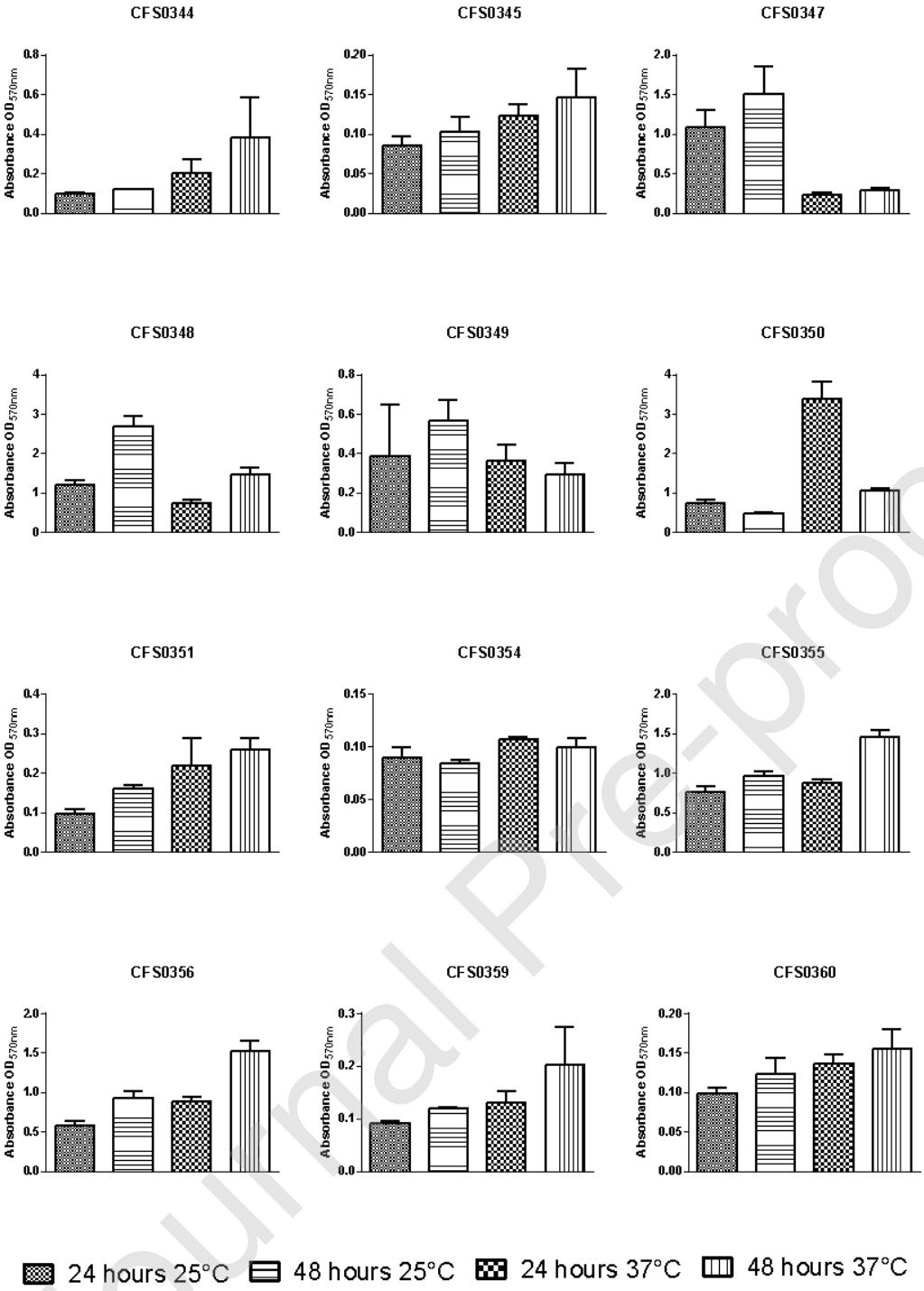

Figure 3. Biofilm biomass quantification for *E. coli* isolates of bovine origin. Biofilms were grown statically in M9 minimal media for 24- and 48-hours at 25- and 37-°C. The biomass formed was subsequently detected by crystal violet staining. Optical densities of 0.46 and 0.32 are references for good biofilm formers at 25 °C and 37 °C respectively.

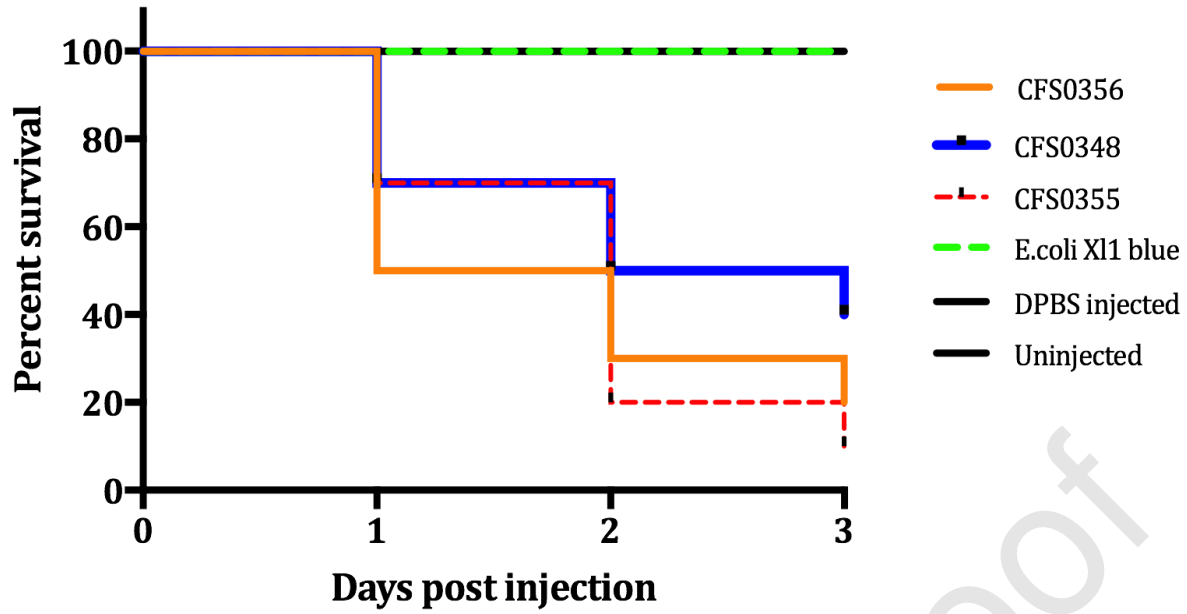

Figure 4. Zebrafish model. Survival curves were generated for zebrafish embryos (n = 30 per *E. coli* strain) infected (100 CFU per embryo) with *E. coli* strains and monitored over 3 days. Zebrafish embryos rapidly succumbed to *E. coli* CFS0355 and CFS0356, while those infected with the *E. coli* CFS0348 died later. Survival curves and trends were significantly different (log_{10}-rank test $p < 0.0001$).

Table 1. A table showing the minimal inhibitory concentrations (MIC) obtained for *E. coli* bovine study isolates, tested against a panel of 10 antimicrobial compounds. AMP - ampicillin; CHL – chloramphenicol; CIP - ciprofloxacin; CN - gentamicin; CTX - cefotaxime; IMP - imipenem; KM - kanamycin; MXF - moxifloxacin; NAL - nalidixic acid; TET – tetracycline. Red coloured values indicate resistant phenotypes.

Escherichia coli	MIC (µg/mL)									
	Amp	CTX	CN	Km	IMP	NAL	CIP	MXF	TET	CHL
CFS0344	>512	1	32	>512	4	>512	16	16	256	256
CFS0345	>512	1	32	>512	4	>512	16	16	256	512
CFS0347	>512	8	16	32	1	>512	8	8	512	128
CFS0348	>512	1	16	>512	1	>512	8	8	512	512
CFS0349	>512	2	1	2	1	>512	4	4	64	512
CFS0350	>512	1	64	>512	2	>512	16	8	256	512

CFS0351	**>512**	1	1	2	1	**>512**	**16**	**8**	**512**	**512**
CFS0354	**>512**	1	1	1	1	**>512**	**8**	**8**	**512**	**256**
CFS0355	**>512**	1	**16**	1	1	**>512**	**16**	**16**	**256**	4
CFS0356	**>512**	1	**8**	2	1	**>512**	**16**	**16**	**256**	4
CFS0359	**>512**	1	1	**512**	1	**>512**	**4**	**4**	**256**	**128**
CFS0360	**>512**	1	1	**256**	1	**>512**	**4**	**4**	**256**	**128**

Table 2. Table summarising features extracted from the whole genome sequences of the *E. coli* collection. The MLST-associated sequence types; the corresponding phylogroup and serotypes; antimicrobial resistance genes organised by classes (determined also phenotypically in **Figure 1**); plasmid incompatibility types; S1 nuclease-PFGE plasmid profiles and genotypes associated with siderophores are included. Positive features are denoted using black squares; white squares indicate absence of marker. Incompatibility types were extracted from the PlasmidFinder 1.3 database. Siderophore sequences were extracted from virulence factor database (VFDB).